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13. ABSTRACT (Maximum 200 Words) This report details our progress during the third year of a three-year proposal. The proposal's overall goal is to uncover biochemical mechanisms that underlie learning and memory. These studies have yielded novel information about the effects of fear conditioning on brain phospholipase C- β 1a (PLC- β 1a), indicating that PLC- β 1a may play an important role in the biochemical processes underlying fear-conditioned learning and memory formation. We have uncovered the molecular basis of the observed association between PLC isozymes and extracellular signal-regulated protein kinase. In addition to increasing our understanding of the biochemical basis of learning and memory, these studies have yielded important information about the neurochemical mechanisms that underlie fear and stress, and, consequently, may provide insight into the neurochemical basis of posttraumatic stress syndrome.				
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A mouse model of prenatal ethanol exposure using a voluntary drinking paradigm.

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Running Title: Learning deficits in FAE mice

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ABSTRACT

Background: The incidence of fetal alcohol spectrum disorders (FASD) is estimated to be as high as 1 in 100 births (Sampson et al. 1997). Efforts to better understand the basis of prenatal ethanol-induced impairments in brain functioning, and the mechanism(s) by which ethanol produces these defects, will rely on the use of animal models of fetal alcohol exposure (FAE).

Methods: Using a saccharin sweetened alcohol solution, we developed a free choice, moderate alcohol access model of prenatal alcohol exposure. Stable drinking of a saccharin solution (0.066%) was established in female mice. Ethanol was then added to the saccharin in increasing concentrations (2%, 5%, 10% w/v) every two days. Water was always available and mice consumed standard pellet chow. Control mice drank saccharin solution without ethanol. After a stable baseline of ethanol consumption (14 g/kg/day) was obtained, females were impregnated. Ethanol consumption continued throughout pregnancy, then was decreased to zero percent in a step-wise fashion over a period of six days after pups were delivered. Characterization of the model included measurements of maternal drinking patterns, blood alcohol levels, food consumption, litter size, pup weight, pup retrieval times for the dams and effects of FAE on performance in fear-conditioned learning and novelty exploration.

Results: Maternal food consumption, maternal care, and litter size and number were all found to be similar for the alcohol-exposed and saccharin control animals. FAE did not alter locomotor activity in an open field, but did increase the time spent inspecting a novel object introduced into the open field. FAE mice displayed reduced contextual fear, when trained using a delay fear conditioning procedure.

Conclusions: The mouse model should prove to be a useful tool in testing hypotheses about the neural mechanisms underlying the learning deficits present in FASD. Moreover, a mouse prenatal ethanol model should increase the opportunity to use the power of genetically defined and genetically altered mouse populations.

Key words: fetal alcohol syndrome, ethanol, mouse, voluntary drinking behavior

The incidence of fetal alcohol spectrum disorders (FASD), which includes Fetal Alcohol Syndrome (FAS) and alcohol-related neurodevelopmental disorder (ARND) (Streissguth and O'Malley, 2000), is estimated to be as high as 1 in 100 births (Sampson et al., 1997). Children with FASD display a variety of cognitive and behavioral aberrations, ranging from severe mental retardation to subtle deficits that become apparent under stressful conditions (Streissguth et al., 1990; Streissguth et al., 1994; Mattson and Riley, 1998). Efforts to better understand the basis of prenatal ethanol-induced impairments in brain functioning, and the mechanism(s) by which ethanol produces these defects, will rely on the use of animal models of fetal alcohol exposure (FAE).

Most studies on fetal alcohol effects have employed the rat using a variety of ethanol administration paradigms and schedules. Prenatal ethanol exposure paradigms commonly either require dams to ingest an ethanol-containing liquid diet or to be intubated, or injected, with ethanol. These procedures produce variable degrees of maternal stress, which, in turn, may modify the effects of prenatal drug exposure on the developing fetus (Ward and Wainwright, 1989; Slone and Redei, 2002). The use of liquid diets also limits the amount of ethanol that can administered without inducing malnutrition. An additional confound associated with many existing paradigms of fetal alcohol exposure (FAE) is that cross-fostering of the pups is employed in order to avoid the introduction of differences in maternal care that may occur between control (non-alcohol consuming) and alcohol drinking moms, whether alcohol availability is continued postpartum, or whether alcohol is withdrawn. The quality of maternal care has been shown to produce significant changes in hippocampal synaptophysin immunoreactivity and Morris water maze performance in the offspring (Liu et al., 2000). Even brief maternal separation (45 min) has been shown to increase the expression of nerve growth factor in the dentate gyrus and hilus regions of the hippocampal (Cirulli et al., 1998).

We sought to develop a mouse model of FAE that minimized these problems. In the present studies, we used saccharin sweetened ethanol solutions (Czachowski et al., 1999; Roberts et al., 1999; Tomie et al., 2002) and a modification of the sucrose fading approach (Slawecki et al., 1997) to establish consistent, voluntary alcohol consumption patterns prior to impregnating female mice, then maintained alcohol consumption throughout gestation. Nutrition was provided using a standard pellet diet. Following delivery of the offspring, step wise decreases in ethanol concentrations in the saccharin solution prevented the display of ethanol withdrawal signs in the mother and eliminated the need to cross-foster the pups. This procedure did not significantly impact various measures of maternal or neonatal well-being, or maternal care for the pups, yet resulted in cognitive and behavioral impairments in the offspring consistent with other models of FAE.

MATERIALS AND METHODS

Ethanol Drinking and Fetal Alcohol Exposure Paradigms.

All of the procedures employed in the current studies were approved by the University of New Mexico Health Sciences Center Laboratory Animal Care and Use Committee.

Sixty day-old B6SJL/F1 (Harlan Industries, Indianapolis, IN) female mice were individually housed in plastic cages in a temperature controlled room (22 °C) on a 12 hr dark: 12 hr light schedule (lights on from 0700 to 1900 hr). Standard chow and water were available ad libitum.

Prenatal exposure of mice to ethanol was performed using saccharin sweetened solutions (Czachowski et al., 1999; Roberts et al., 1999; Tomie et al., 2002). Female mice were offered 22 hr free access to either 0.066% saccharin or water for 2 weeks. Subsequently, ethanol was added to the saccharin tube for the experimental groups, while the control group continued to have access to saccharin alone. The concentration of ethanol was increased in a step wise fashion every two days, from 0% to 2%, to 5%, then, finally, to 10% (w/v). After two weeks of drinking, a male was introduced into the female's cage. Once the female was determined to be pregnant by the presence of vaginal plug, the male was removed and nesting material was placed in the cage. Ethanol consumption was not measured for the 1-2 days while the male was present in the cage. Females continued to drink stably throughout pregnancy. Within one day of birth, the alcohol and the saccharin concentrations were reduced by one-half every two days, until the mice were consuming only water. Saccharin consuming mothers were weaned off of the sweetened water in a similar step down fashion. Litter sizes and litter weights were determined on postnatal day (PND) 7. Pups were weaned at 23 days and maintained in same sex, litter-mate housed cages with free access to water and chow. Offspring (both male and

female) were 60-100 days old, when used in the present experiments. No more than two littermates were represented in any single treatment condition.

Characterization of Maternal Care

Maternal grooming (frequency and duration) and care for the pups (latency to retrieve pups removed from the nest and time spent on the nest) were evaluated at PND 3-5 between 1000 and 1300 hr by videotape monitoring.

Blood Ethanol Measurement

Maternal blood ethanol concentrations produced by ad libitum consumption of the 10% ethanol/0.066% saccharin solutions were measured using saphaneous vein puncture (a 10 μ L sample) and determined enzymatically (Farr et al., 1988). Blood samples were collected from nine separate groups of mice at 15 different times between 0000 and 2400 hr during the second week of gestation; each dam gave a maximum of three different samples. Blood alcohol was determined for three separate breeding rounds of FAE animals. Since restraint and blood taking are stressful, these measures were performed on a separate group of dams who were drinking at a rate similar to the experimental dams; the pups from these dams were not used in these studies.

Whole blood was collected from the saphenous vein and immediately mixed with 0.2 mL of 6.6% perchloric acid and stored frozen at -20 °C until assayed. Blood ethanol standards were created by mixing whole blood from untreated mice with known amounts of ethanol ranging from 0 to 240 mg/dL and then mixing 0.1 mL aliquots of each standard with perchloric acid and storing the standards frozen with the samples. Blood ethanol samples were assayed using a

modification of the method of Lundquist (1959). The ethanol standard curve was linear over the 0-240 mg/dL range. Sample blood ethanol values were determined by regression analysis.

Corticosterone Radioimmunoassay and Adrenal Weights

After obtaining body weights, adult mice were rapidly decapitated, blood was collected, and the adrenal glands were dissected, separated from the capsule and wet weighed. Trunk blood was collected into chilled tubes containing EDTA (7.5 mg) and aprotonin (0.2 mL/0.5 mL blood) and centrifuged at 2,200 x g for 10 min at 4 °C. Supernatant (plasma) was then collected into clean tubes and stored at -80 °C until used in the assay. Corticosterone levels were analyzed in duplicate using a mouse corticosterone RIA kit (ICN Biochemicals, Costa Mesa, CA). The detection limit for the kit was determined to be 3 ng of corticosterone/mL with a within assay variance of 4.5% and a between assay variance of 6.5%.

Novel Object Exploration

The novel object test was adapted from the protocol previously described by Grailhe et al. (1999). An open field apparatus, measuring 17" x 17" with 8" high Plexiglas side-walls, was used. The floor of the apparatus was black and divided into five areas: four equal quadrants and one center area having a diameter of 14 cm. The experiments were carried out in a dimly lit room with the aid of a video camera to minimize effects of stress and anxiety. The floor and walls were wiped with 70% isopropanol before each test session. The test session consisted of two 5 minute periods. At the start of the first five-minute session, mice were placed into the center area and the latency to leave the center, the total time spent in the center and the total number of center line crosses and the number of total lines crossed were recorded as measures of exploration. At the start of the second five-minute period, a pink and green striped gray cube, one inch³, with an open side was placed into the center of the

apparatus, with the open side facing the mouse. Measurements were made of the latency to approach the novel object, the total time spent in the center area, the total number of center line crosses and the number of total line crosses.

Delay Fear Conditioning

Fear conditioning was conducted using a procedure similar to that described by Wehner and colleagues (Lu and Wehner, 1997; Smith and Wehner, 2002). A Coulbourn Instruments (Allentown, PA) Habitest® System with two metal walls, two Plexiglas walls, and a stainless steel grid floor for administration of the foot shock was used for conditioning. The apparatus was located within a sound-attenuated chamber.

Mice were trained as follows. After 90 seconds of habituation in the conditioning context, the conditioned stimulus (CS), an 80 dB, 6 Hz clicker, was initiated. The unconditioned stimulus (US), an electric foot shock (0.55 mA), was delivered during the last two seconds of the 30 second CS. Ninety seconds later, the CS-US sequence was repeated. Thirty seconds after co-termination of the second CS-US pairing, the animal was removed from the conditioning context and returned to its home cage.

Approximately 24 hours later, mice were assessed for their conditioned responses to the context and to the CS by measuring freezing (defined by the absence of movement other than those necessary for respiration; Bouton & Bolles, 1980; Fanselow, 1980) behavior following reintroduction into the context in which conditioning occurred and exposure to the CS in a novel context (a clear plastic container with orange scented bedding). Freezing was measured using a time-sampling procedure in which every 10 seconds the mouse was scored as either moving or freezing. The amount of freezing (expressed as a percentage) was calculated by dividing the number of observations periods where the mouse was frozen by the total number of

observation periods. Freezing in the conditioning context (in the absence of the CS and US) was measured for four minutes. Freezing to the CS in the novel context was determined approximately one hour after assessment of contextual conditioning. Basal levels of freezing in the novel context were scored for three minutes without presentation of the CS. After basal scoring, the CS was initiated and remained on while freezing was scored for an additional three minutes.

RESULTS

Effect of saccharin fading ethanol exposure paradigm on food and fluid consumption.

We monitored the consumption of standard breeder chow and water as the ethanol was introduced. While there was a small increase in food consumption upon introduction of the saccharin (Figure 1A), there were no significant alterations in either the eating (Figure 1A) or drinking (Figure 1B) patterns of the mice. Figure 1B shows that the gradual introduction of ethanol was readily accepted by the mice, with a slight reduction in intake once the 10% (w/v) ethanol was introduced into the bottles. Saccharin drinking control moms maintained a stable level of preference for the saccharin across their pregnancy (Figure 2), drinking almost exclusively from the saccharin-sweetened water tubes. Similarly, the 10% (w/v) ethanol drinking moms developed a stable preference for the sweetened ethanol solution prior to pregnancy and maintained this preference throughout the pregnancy (Figure 2). It should be noted that, while there is a clear preference for the sweetened solutions, the mice reduced their water intake, such that there was no significant increase in their total fluid consumption.

Effect of saccharin fading ethanol exposure paradigm on maternal blood alcohol levels.

Blood alcohol levels, measured in a separate group of ethanol drinking dams were evaluated over a 24 hour period (Figure 3). Peak blood alcohol levels were achieved between 1600 hr and 0100 hr, during the dark cycle. The average daily consumption of ethanol by mouse dams on the 10% ethanol saccharin solutions was 14.0 g EtOH/kg body weight/day, generating average peak blood alcohol level of 120 (\pm 9) mg/dL consistently throughout the pregnancy (Figure 3). The average calorie equivalent of the ethanol consumed was about 10% the daily diet intake.

Effect of saccharin fading ethanol exposure paradigm on pup weight, litter size and maternal weight.

Food consumption (Table 1) was monitored every other day and no difference between the saccharin sweetened ethanol and saccharin alone drinking females in average daily food consumption was noted. Additionally, there was no significant difference in the change of maternal weight from Day 1, the start of 10% (w/v) ethanol, to Day 17, the day male was introduced into the cage. Average pup weights and litter sizes did not significantly differ between the ethanol and saccharin drinking groups (Table1). No gross anatomical abnormalities were noted at birth in the fetal ethanol-exposed mice.

Effect of saccharin fading ethanol exposure paradigm on maternal grooming and latency to retrieve pups.

While pup weights and growth are good indicators of maternal care, we also addressed maternal care using a pup retrieval test, as well as monitoring grooming frequencies over 4 one-hour observation sessions (Table 2). The ethanol drinking moms retrieved pups that were deliberately removed from the nest with a similar latency to that of the saccharin drinking moms (Table 2). Further, ethanol consuming females did not differ from controls in the percent time spent on the nest or grooming of pups.

Effect of saccharin fading ethanol exposure paradigm on offspring basal corticosterone levels

Several studies have shown that rats prenatally exposed to ethanol demonstrate changes in hypothalamic-pituitary-adrenal responsiveness, as indicated by elevations of adrenocorticotropin and corticosterone, especially after stress (Glavas et al., 2001; Osborn et al, 1996). Levels of corticosterone in blood were determined without using any activating stress other than handling the mouse just prior to decapitation. In our study, FAE females had a

significantly higher plasma corticosterone level than did control females (Table 3). Although a similar difference was observed for males, it did not achieve significance. This was demonstrated by a significant interaction of sex x prenatal treatment condition interaction ($F(1,28) 5.67, p<0.03$). No differences were observed in adrenal weights of FAE and control offspring.

Effect of saccharin fading ethanol exposure paradigm on novelty exploration.

Exploratory behavior within the testing environment (initial 5 minute segment, without object present) and exploratory behavior in response to introduction of a novel object (second five minute segment, with novel object) were assessed by recording the number of transitions, time spent in center and number of entries into the center area (Figure 4). In these studies adult male offspring were used. Data were analyzed by analysis of variance with Bonferroni correction. Prenatal condition (ethanol versus saccharin) did not significantly affect the number of entries into the center, regardless of the object presence (Figure 4,B); thus, this dependent variable was not analyzed by ANOVA. There was no effect of prenatal condition on the number of transitions (Figure 4C), but a significant decrease in number of transitions occurred when the object was present for both of the prenatal treatment conditions [$F(1,12)=17.3, p<0.01$]. This suggested that there was no significant difference between the prenatal treatment groups in general locomotor activity under these conditions. Time spent in the center with the object present was significantly greater for the FAE mice (Figure 4A), as indicated by the significant interaction between prenatal condition and object presence [$F(1,12)= 169.58 p<0.0001$], suggesting greater exploratory activity.

Effect of saccharin fading ethanol exposure paradigm on delay fear conditioning.

FAE mice and control pups were reared to adulthood and trained using a delay conditioning paradigm. Twenty-four hours later, mice were assessed for their conditioned responses to

the context and to the CS by measuring freezing behavior in response to reintroduction into the context in which conditioning occurred and exposure to the CS in a novel (altered) context (Figure 5). As can be seen in Figure 5, FAE mice froze less to the context than did saccharin controls, while freezing to the auditory CS was nearly identical in the two groups. An ANOVA revealed a significant effect of prenatal treatment condition [$F(1,36)=18.3$, $p<0.001$] and a significant interaction between prenatal treatment and stimulus condition (tone versus context) [$F(1,36)=19.5$, $p<0.001$], indicating the FAE mice froze less to the context than saccharin mice but there were no differences between the prenatal treatment conditions in freezing to the tone.

DISCUSSION

Our goal was to develop a prenatal alcohol exposure model that would: 1) produce a voluntary and stable drinking of moderate concentrations of ethanol throughout pregnancy, 2) not significantly affect maternal care, and 3) replicate the behavioral effects found previously in the rat moderate alcohol drinking model. We believe that we have developed a mouse model of FAE that accomplishes these goals. Using a moderate dose of ethanol (10%, w/v), this paradigm did not affect fetal birth weight, litter size, maternal care, or locomotor activity of the offspring, yet, characterization of the behavioral phenotype of the offspring revealed deficits in contextual fear conditioning, as well as increased novelty exploratory behavior. In addition, we found no effect on pup mortality rates. This is in sharp contrast to earlier mouse models which reported a marked decrease in the number of viable pups among the alcohol diet groups (Boggan et al., 1979; Randall and Taylor, 1979; Fish et al 1981).

The paradigm described herein developed drinking behavior in females prior to impregnation. Thus, stable drinking patterns and blood levels were established before the beginning of pregnancy. We believe that this more closely models human alcohol drinking patterns than do paradigms in which alcohol consumption is initiated once pregnancy has been established.

An additional goal of our model was to permit the mothers to care for their own pups, thereby reducing the stress of cross-fostering. In order to accomplish this, we needed to reduce the alcohol concentration after the pups were delivered as quickly as possible, but without precipitating any withdrawal signs. In several pilot studies we determined that reducing both the ethanol and saccharin concentrations by one half every two days was slow enough to prevent any signs of handling-induced seizures or maternal neglect. While gross measurements, such as pup retrieval times, pup weights, pup grooming and litter sizes, suggest that maternal care was appropriate; other subtle differences between 10% ethanol and

saccharin drinking mothers are likely to exist. In humans, it is well known that maternal alcohol consumption may slightly reduce milk production and that some of the alcohol consumed is transferred in the milk (for review, Mennella, 2001). Using a rat model of prenatal and postnatal 20% alcohol exposure, Murillo-Fuentes et al. (2001), found no significant effect of ethanol on birth weight, but milk consumption and suckling behavior were reduced in pups whose mother continued to consume 20% ethanol. In our model, the nursing mothers were tapered off the ethanol solution and this may be why we did not see a significant difference in the pup weight between the 10% ethanol and saccharin drinking control groups.

Performance in the novel object task is an indication of exploratory responsiveness to novel stimuli, and, while it is not a standard measure of anxiety, results of this task are influenced by anxiety. The test begins similar to a standard open field, spontaneous locomotion study, where entries into the center of the field are suggestive of lower levels of anxiety in the mouse. Mice generally display neophobia and, though attracted by novel objects, typically keep a safe distance from them (see File, 2001 for review). Mice that perform with reduced anxiety on other tests (e.g., elevated plus and social interaction) typically will spend more time in close proximity of the novel object. An increase in number of entries into close proximity to a novel object indicates increased inquisitive behavior, while increased time spent near the novel object indicates inspective behavior (Grailhe et al. 1999; Dellu et al 2000). In our study, the 10% ethanol mice reacted to the presentation of a novel object with increased inspective behavior compared to saccharin animals. It is not likely that the increase in time spent in proximity to the novel object was due to decreased locomotor activity, since entries into the center and the number of transitions in the open field were not different in the 10% ethanol and saccharin mice. The higher level of inspective behavior may be due to a failure in the formation of associations and information processing, as suggested by their impaired performance in the

fear conditioning task (see below). Another interpretation of these data is that the 10% ethanol mice display a reduced level of anxiety.

The hippocampal formation has long been a focus of studies attempting to uncover the basis of ethanol-induced learning and memory deficits. In rodents, FAE is associated with impaired learning and/or memory in a variety of hippocampal-dependent tasks, including: radial arm maze paradigms (Reyes et al. 1989), the Morris water maze task (Gabriel et al. 2002, Savage et al. 2002), and avoidance learning (Furuya et al. 1996). In a recent group of studies, we found that rats that were exposed prenatally to moderate levels of ethanol display deficits in contextual fear conditioning, but not fear of a discrete CS (Weeber et al. 2001). Similar to the findings of Weeber et al., (2001) using the rat model, the 10% ethanol mice displayed significant deficits in single trial fear conditioning (Figure 5). However, with the mouse model, a more robust attenuation of fear conditioned freezing was seen here (Figure 5) compared to that reported in rat (Weeber et al., 2001). Impaired contextual conditioning, but unaltered CS (tone) conditioning, indicates that FAE disrupts hippocampal functioning, while leaving amygdalar functioning intact. The hippocampal formation plays a critical role in the consolidation and expression of contextual fear memory, whereas the amygdala plays an essential role in the acquisition and consolidation of information about both the elemental CS and the training context, as well as the expression of both CS fear and contextual fear responses (see Weeber et al 2001 for discussion). These results indicate that the 10% ethanol mice have impaired formation of the association between the context and the US. Identification of a learning/memory deficit in adult offspring demonstrates that the mouse, as well as the previously described rat, FAE model produces learning and memory impairments which persist into adulthood, similar to the clinical course of FASD in humans. It is interesting to note that Green et al. (2002) reported that eyeblink delay conditioning is

impaired in rats that were exposed to alcohol as neonates, approximating the human third trimester period. Thus, FAE-induced deficits in associative learning measured using delay conditioning paradigms are reproducible across species and appear to be a useful behavioral endpoint for the study of the effects of therapeutic interventions on FAE-induced learning and memory deficits.

An advantage of this mouse paradigm is that it will support drinking of high, moderate and low ethanol amounts in a voluntary design. The procedure that we describe could be modified to study effects of "binge"-like drinking, as we have found that, by limiting alcohol availability to a two-hour period, females will display higher alcohol drinking. For example, we have found that mothers will drink saccharin sweetened 15% (w/v) ethanol in a two hour restricted access paradigm and generate blood alcohol levels of 150-180 mg/dL. In addition, the use of a voluntary drinking approach can be employed for the study of the effects of high blood alcohol levels as it avoids the problems of malnutrition that are a consequence of using higher concentrations of ethanol in liquid diets or the stress associated with oral intubation, which is often employed to administer larger quantities of ethanol to dams.

The mouse FAE model described herein should prove useful in studies that exploit the availability of genetically modified, as well as several well characterized, mouse strains to assess the interplay between different genes in determining susceptibility to and the outcome of FAE. The studies presented here were performed using B6SJL/F1 mice because of our interest in developing a model that could be used with genetically altered mouse populations. However, it was important to demonstrate that similar results could be obtained in a genetically stable inbred strain. In a series of pilot studies we have obtained similar results on drinking levels, litter sizes and pup weights using the C57 BL/6 J mice (data not shown). It is important

to note that, though the mouse does offer certain important advantages over other animal models of FAE, it will be important to study outcomes in a variety of models.

Finally, it is possible that one model may more closely replicate certain characteristics of human FASD (e.g., attention deficits), while another model more closely reproduces other characteristics (e.g., impaired spatial learning). Extensive characterization of the effects of FAE on various neurochemical, electrophysiologic and behavioral endpoints in other models has identified several targets for therapeutic intervention, which could be tested in our mouse model. Further, thorough characterization of the effects of FAE in different animal models may identify distinct endpoints, which, in light of known differences in rodent brain neurochemistry and neurophysiology (e.g., differences in hippocampal GAP-43 expression, McNamara et al, 1996), may provide clues to the effects of genetic influence on the effects of FAE, and, thus identify therapeutic targets.

In conclusion, we describe the development and initial characterization of a mouse model of FAE. This model complements and extends other available models for the study of FAE. The paradigm that we employed will facilitate the study of the impact of FAE without negatively influencing diet, natural consumption routes, or requiring surrogate maternal care, all of which are thought to contribute to maternal stress. The mouse FAE model allows the use of genetically altered mice, as well as genetically defined inbred mouse strains, for identifying risk factors that are associated with FASD cognitive deficits.

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TABLE 1

Maternal weight change, food consumption, pup weights, litter size, from 4 separate studies
(Values are mean \pm SEM, n= 6 mothers per study)

Treatment Condition	Change in maternal weight (g)	Daily food consumption (gm)	Pup weight (g)	Litter size
10% (w/v) EtOH in saccharin	+1.08 (\pm 0.2)	4.4 (\pm 0.38)	4.3 (\pm 0.4)	8.4 (\pm 1.3)
Saccharin only	+1.02 (\pm 0.4)	4.2 (\pm 0.13)	4.1 (\pm 0.2)	8.2 (\pm 2.0)

TABLE 2

Pup retrieval time, percent time nesting and pup grooming from 4 separate studies (Values are mean \pm SEM, n= 6 mothers per study)

Treatment Condition	Pup retrieval time (sec)	Percent time on nest (min/2hour observation)	Percent time grooming pup
10% (w/v) EtOH in saccharin	62 (± 8)	52 (± 3)	7.2 (± 1.0)
Saccharin only	57 (± 1)	49 (± 4)	9.7 (± 0.9)

TABLE 3

Average adrenal weights and plasma corticosterone levels in offspring from saccharin and ethanol drinking dams taken at 0900-1000 hr. Values are mean (\pm SEM, n=10). Asterisk indicates statistically different from saccharin females, $p < 0.03$.

	Average single Adrenal Weight (mg)	Total Adrenal wt/ body wt x 100	Plasma Corticosterone level ug/dl
Saccharin female	3.10 (\pm 0.25)	0.0261 (\pm 0.003)	3.00 (\pm 0.4)
10% EtOH female	2.74 (\pm 0.20)	0.0253 (\pm 0.004)	5.06 (\pm 0.4) *
Saccharin male	1.46 (\pm 0.18)	0.0196 (\pm 0.009)	2.78 (\pm 0.3)
10% EtOH male	1.35 (\pm 0.21)	0.0163 (\pm 0.006)	3.30 (\pm 0.4)

FIGURE LEGENDS

FIGURE 1

The effect of the saccharin fading ethanol exposure paradigm on food and fluid consumption.

In panel A, the amount (mean \pm SEM, n=12) of food consumed by the female mice assigned to the ethanol drinking condition is presented. Consumption of the standard breeder block chow was measured every day for a total of 10 days while saccharin and ethanol were introduced.

Panel B presents the volumes (mean \pm SEM, n=12) of fluid consumed from the water (square symbols) and saccharin or saccharin/ethanol tubes (triangle symbols) for a total of 10 days for the same set of female mice.

FIGURE 2

The effect of the saccharin fading ethanol exposure paradigm on fluid consumption during pregnancy.

Figure 2 presents the two day total amount (mean \pm SEM, n=12) of water only (squares) and total fluid (triangles) consumed by pregnant dams in the saccharin drinking group (filled symbols) and the 10% w/v ethanol drinking (open symbols) conditions. Water and total fluid consumption (two day totals; mean \pm SEM, n=12) are given from day 2 – day 14 of their pregnancies.

FIGURE 3

Effect of saccharin fading ethanol exposure paradigm on blood alcohol levels

Blood alcohol level determinations from saphenous vein blood of dams drinking 10% (w/v) ethanol in 0.06% saccharin across a 24 hour access period. Each point is mean \pm SEM from

6-8 dams per time point. Each dam is represented at no more than 3 time points. Solid horizontal bar indicates the lights on period.

FIGURE 4

Effect of saccharin fading ethanol exposure paradigm on novelty exploration.

Novelty exploration was measured in adult male mice and expressed as mean (\pm SEM, $n=7$ per group) time spent in the center (within 5 cm of the object, panel A), number of entries into the center area (panel B) and number of transitions across the quadrants of the open field (panel C). Data are presented as activity during the first 5 minutes without the object present in the first pair of bars and the behavioral activity during the last 5 minutes while the novel object was present in the last pair of bars. Asterisk represents $p<0.01$ by ANOVA.

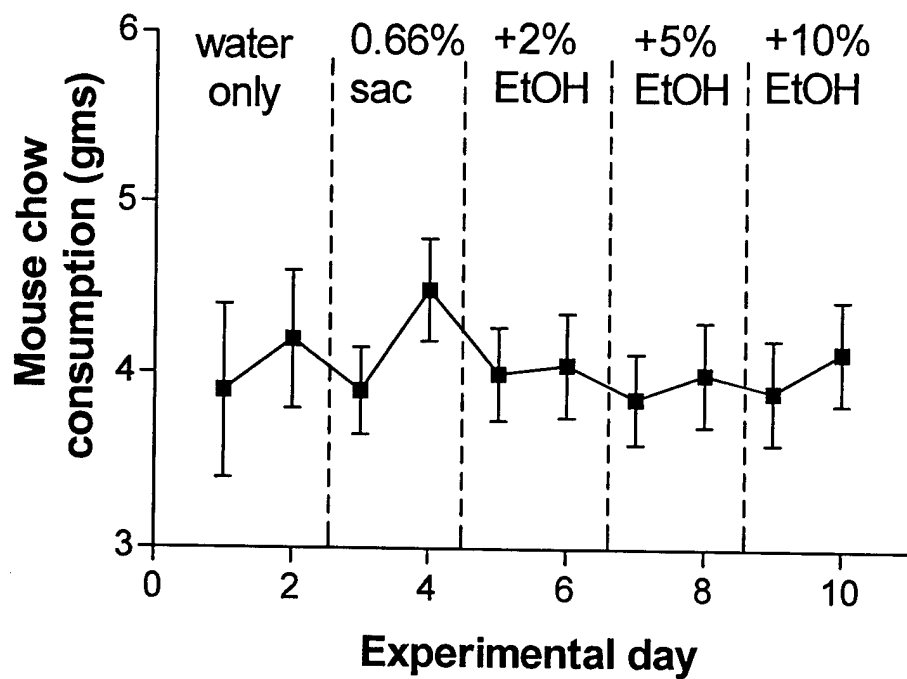
FIGURE 5

Effect of saccharin fading ethanol exposure paradigm on contextual fear learning and memory.

Freezing to the training context (top) and to the conditioned stimulus in a novel context (bottom) in saccharin control and 10% EtOH mice trained with a paired tone-shock. Adult offspring of dams consuming either 0.066% saccharin (Control; $n=10$) or 10% (w/v) ethanol in 0.066% saccharin (10% EtOH; $n=10$) were trained using a delay fear conditioning paradigm. Panel A : Twenty-four hours after training, the animals were returned to the training context and the conditioned response (freezing) was assessed. Data are expressed as the % freezing ($\#$ of freezing intervals \div total intervals). Panel B: Freezing to the CS tone was measured in a novel context. Data are expressed as described above. Asterisk represents $p<0.001$ by ANOVA.

FIGURE 1

A.



B.

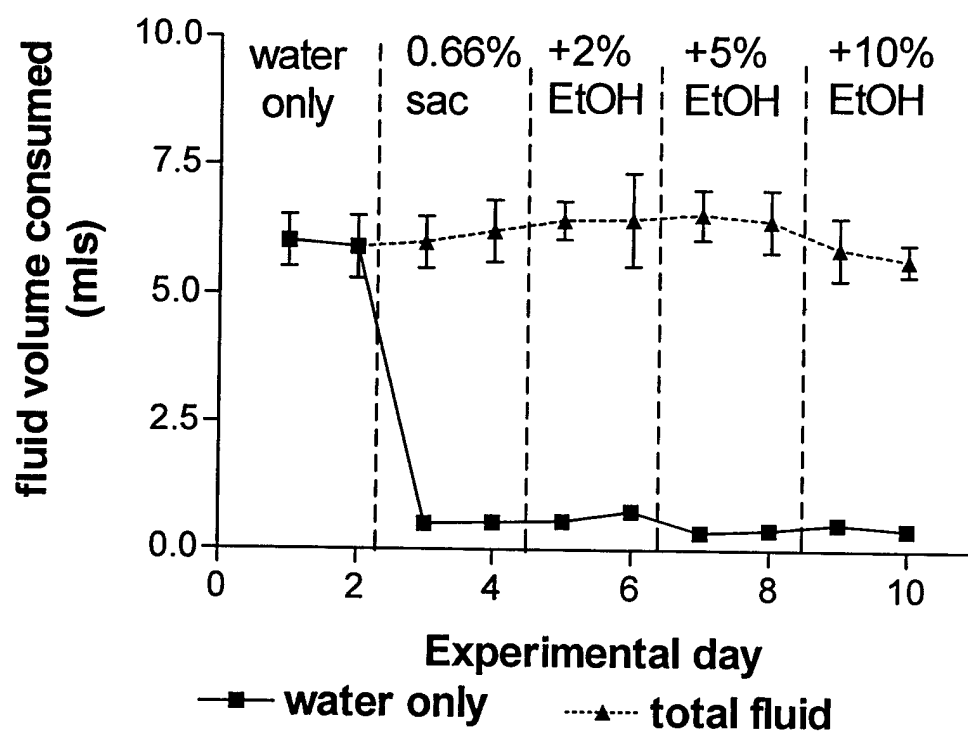


FIGURE 2

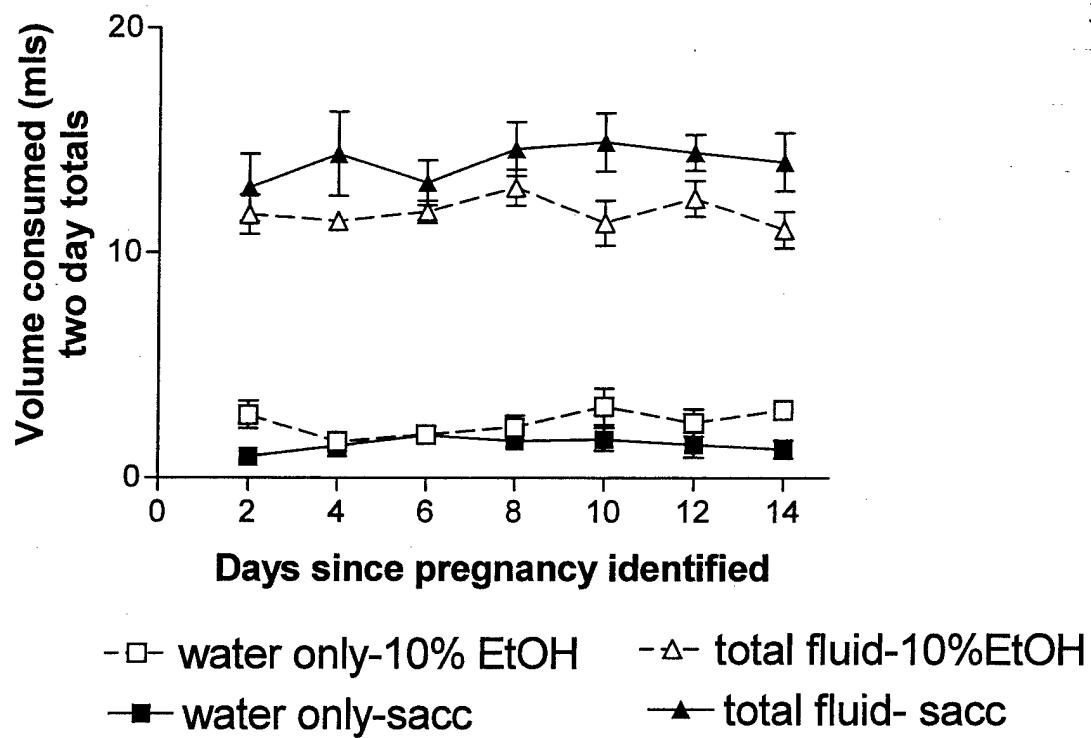


FIGURE 3

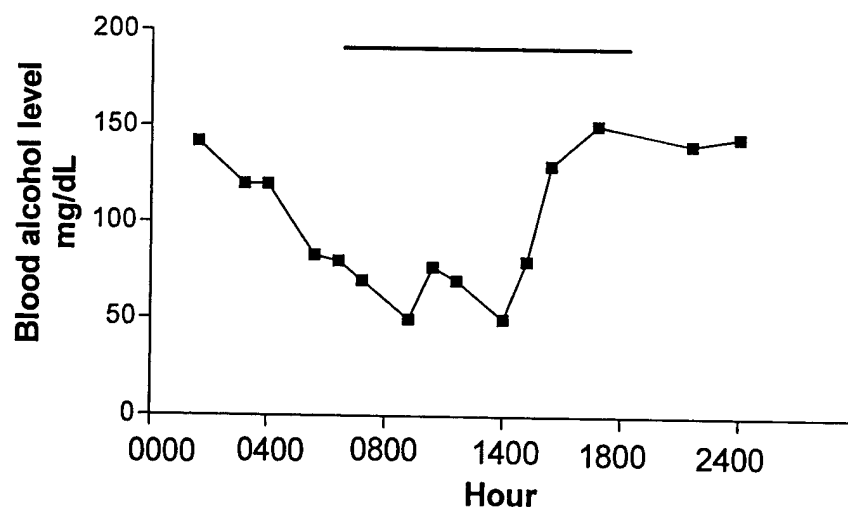


FIGURE 4

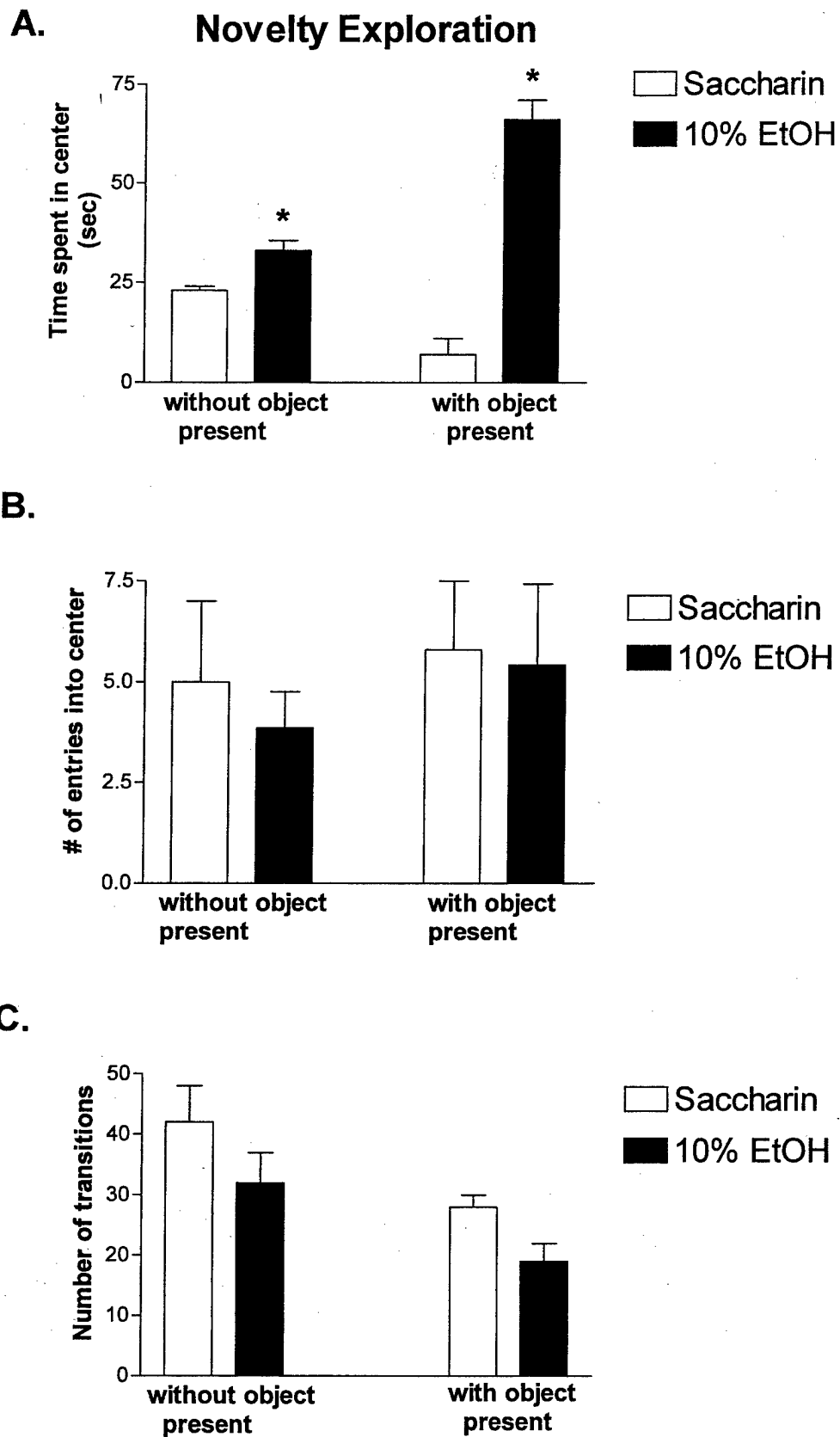
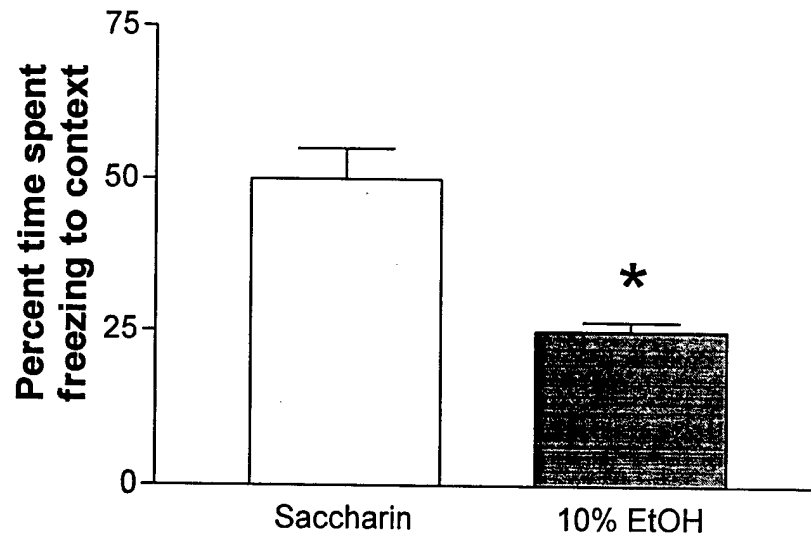


FIGURE 5

A.



B.

